Center for Veterinary Biologics and National Veterinary Services Laboratories Testing Protocol

Supplemental Assay Method for Testing Growth Promoting Qualities of Fluid Thioglycollate Medium and Soybean-Casein Digest Medium using *Bacillus subtilis* Spores and *Candida krusei* as the Indicator Organisms

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Contact Person: Gerald G. Christianson (515) 663-7416

Approvals:

/s/ Gerald G. Christianson Date: 3/22/99
Gerald G. Christianson, Head
Cytology/Sterility Section

/s/ P Frank Ross Date: 22 Mar 99
P. Frank Ross, Acting Quality Assurance Manager

/s/ Randall L. Levings Date: 3/22/99
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

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Table of Contents

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1. Introduction

This is a Supplemental Assay Method (SAM) for testing Fluid Thioglycollate Medium (FTM) and Soybean-Casein Digest Medium (SCDM) for growth promoting qualities, as required in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.25(b).

2. Materials

2.1 Equipment/instrumentation

2.1.1 30º-35ºC incubator

2.1.2 20º-25ºC incubator

2.1.3 Sterile disposable cotton-plugged pipettes

2.1.4 Sterile 10-ml disposable syringes with needles

2.1.5 Biosafety cabinet

2.1.6 Magnetic stirrer

2.2 Reagents/supplies

2.2.1 Indicator Organisms: Use *Bacillus subtilis* (ATCC #6633) and *Candida krusei (*ATCC #6258) (or equivalent organisms as specified in the current United States Pharmacopoeia (USP) as the control organisms in order to determine the growth promoting qualities of the medium according to 9 CFR, Part 113.25.

2.2.2 Media: Brain Heart Infusion Agar (BHIA), Soybean-Casein Digest Agar (SCDA) or Trypticase Soy Agar (TSA), SCDM or Trypticase Soy Broth (TSB), FTM. See **Section 9.1** for media formulations.

2.2.3 Stabilizer: Phosphate buffered saline (PBS) with 12% sucrose (**Section 9.1.5**)

2.2.4 Sterile glycerin

3. Preparation for the Test

3.1 Personnel qualifications/training

The personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. The personnel must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the Center for Veterinary Biologics-Laboratory (CVB-L) or equivalent, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

3.2.1 Turn on biosafety cabinets at least 1 hour before preparing positive control reagents or testing media for growth promotion.

3.2.2 Monitor incubators daily for temperature according to the current version of NVSLSOP0001.

3.2.3 Monitor freezers and coolers daily for temperature according to the current version of NVSLSOP0003.

3.3 Preparation of *Bacillus subtilis* reagent

3.3.1 Place 1 ml of *Bacillus subtilis* spore suspension from previous stock culture into a 500-ml flask containing 100 ml of BHIA.

3.3.2 Swirl flask to wet entire agar surface.

3.3.3 Incubate flask at 30°-35°C for 7 days. Make a Gram stain of the resulting growth and examine microscopically for purity.

3.3.4 On the 7th day place about 2 dozen sterile glass beads and a 160-ml solution of 50% SCDM and 50% glycerin in the flask.

3.3.5 Swirl the flask until the glass beads have loosened most of the growth from the agar.

3.3.6 Pour this solution into a sterile 250-ml flask.

3.3.7 Incubate this flask at room temperature (20°-25°C) for 7 more days.

3.3.8 After the second 7 days, place 1.5-ml aliquots of this stock culture (spore suspension) into small sterile 2-ml serum vials and freeze at -70°C.

3.4 Preparation of *Candida krusei* reagent

3.4.1 Inoculate 2 petri dishes containing BHIA with 1 loopful of *C. krusei* from previous stock culture.

3.4.2 Incubate at room temperature (20°-25°C) for 7 days. Make a Gram stain of the resulting growth and examine microscopically for purity.

3.4.3 Take 1 loopful (10 µl) of a colony on the BHIA and inoculate a 250-ml flask containing 120 ml of SCDM.

3.4.4 Incubate the flask of SCDM for 24 hrs at 30°-35°C.

3.4.5 Check the flask of SCDM for purity by Gram stain.

3.4.6 Pipette 1 ml of the SCDM containing the *C.* krusei onto 100 ml of SCDA in a 500-ml flask (inoculate 3-5 flasks). Swirl the flasks to spread the culture for even growth.

3.4.7 Incubate these SCDA flasks for 5-7 days at 20°-25°C.

3.4.8 Pour 150 ml of PBS with 12% sucrose (**Section 8.1.5**) and about 2 dozen glass beads into each flask of SCDA and then swirl the flasks to remove the growth from the agar surface.

3.4.9 Recheck each flask for purity by Gram stain.

3.4.10 Combine the PBS with 12% sucrose from all flasks into 1 flask.

3.4.11 Place this flask on a magnetic stirrer for 1 hr.

3.4.12 Filter the PBS with 12% sucrose through sterile gauze to remove the glass beads.

3.4.13 Place 1.5 ml of the PBS with 12% sucrose into 2-ml serum vials, lyophilize, and store at -70°C.

4. Performance of the test

4.1 Establishing the dilutions to be used to test new media

4.1.1 Remove a vial of the newly prepared stock culture from the freezer and rapidly thaw or rehydrate the culture with 1 ml of SCDM.

4.1.2 Make tenfold dilutions of the stock culture by using a 1-ml pipette to place 1 ml of the stock culture in 9 ml of SCDM (10^{-1} dilution).

4.1.3 Mix by inverting the tube several times.

4.1.4 Using a 1-ml pipette, transfer 1 ml of 10^{-1} culture dilution to 9 ml of SCDM (10^{-2} dilution). Mix as before and continue the procedure until 10^{-10} dilution is prepared.

4.1.5 Incubate the *B. subtilis* dilution tubes for 24-48 hr at 30°-35°C. Incubate *C. krusei* 14 days at 20°-25°C.

4.1.6 Examine the tubes visually for growth to establish the growth endpoint of the stock culture.

4.1.7 After establishing the growth endpoint, use this dilution and the next lower dilution for testing the new media.

4.1.8 Next, data must be accumulated to confirm these dilutions (**Section 4.1.7**) as the proper ones for testing new media with this stock culture. To gather this data, test 10 batches of each media (FTM) and (SCDM) with 10 tubes per dilution or 5 batches of each

media with 20 tubes per dilution. Use a separate vial of stock culture for each batch.

4.1.9 Growth is expected in 9 or 10 tubes inoculated with the lower dilution (**Section 4.1.7**) and in less than 10 tubes inoculated with the higher dilution (**Section 4.1.6**).

4.1.10 If all of the test batches (**Section 4.1.8**) give the expected number of tubes with growth (**Section 4.1.9**), then these dilutions will be used in testing new media. If not, higher or lower dilutions are confirmed for testing new media as outlined in **Sections 4.1.8** and **4.1.9**.

4.2 Testing the media

4.2.1 Test each batch of SCDM and FTM prepared for Sterility testing for growth promoting qualities with both the *C. krusei* and *B. subtilis* stock cultures. Thaw the frozen vials of stock culture rapidly. Rehydrate with SCDM those stock cultures that are lyophilized. Transfer 1 ml of each stock culture to 2 separate tubes with 9.0 ml of SCDM (10^{-1} dilution). Use a sterile pipette for each transfer.

4.2.2 Mix the 10^{-1} dilution by shaking.

4.2.3 Transfer 1.0 ml of the 10^{-1} culture dilution to 9.0 ml of SCDM (10^{-2} dilution) using a sterile 1-ml pipette. Mix as before and continue until the last 2 dilutions.

4.2.4 Use the last 2 dilutions (established in **Sections 4.1.6 and 4.1.7**) to inoculate the media being tested for growth promotion. Transfer 3 ml of the previous dilution into the first of the end dilutions containing 27 ml of SCDM. Mix as before and transfer 3 ml of this 30 ml to 27 ml of SCDM to give the end dilution. Repeat **Sections 4.2.3** through **4.2.5** for the second stock culture.

4.2.5 Using a sterile 10-ml syringe with needle, deposit 1.0 ml of the last dilution into each of 10, 25 x 200-mm tubes containing 40 ml of SCDM. Refill the same syringe with the same dilution of culture and

deposit 1.0 ml into each 10, 25 x 200-mm tubes containing 40 ml of FTM.

4.2.6 Using the same 10-ml syringe, deposit 1.0 ml of the next lower dilution of culture into each of 10, 25 x 200-mm tubes containing 40 ml of SCDM and into each of 10, 25 x 200-mm tubes containing 40 ml of FTM. Repeat **Sections 4.2.6** and **4.2.7** for the other stock culture.

4.2.7 Incubate all tubes (40) containing the *C. krusei* culture at 20º-25ºC and observe for growth of the organism throughout a 14-day incubation period.

4.2.8 Incubate all tubes (40) containing the *B. subtilis* culture at 30º-35ºC and observe for growth of the organism throughout a 7-day incubation period.

5. Interpretation of the test results

Growth is expected in at least 9 or 10 tubes inoculated with the lowest dilution of each of the organisms and in less than 10 out of 10 tubes inoculated with the next higher dilution of each organism. If the stock culture has not deteriorated and if at least 9 or 10 out of 10 tubes inoculated with the lower dilution of stock culture contain growth, the growth promoting quality of that medium is satisfactory. If less than 9 out of 10 tubes at the lower dilution have growth, then the growth promoting qualities of the media are in question and the test must be repeated. If after repeating the test the media’s growth promoting properties are still in question, the media must not be used and all tests already conducted with this media must be considered no tests.

6. Report of test results

Record the results of these growth promotion tests in the positive control log book next to the media control number for that batch of media.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.25, U.S. Government Printing Office, Washington, DC, 1998.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. The following is a list of significant changes made from the superseded protocol:

8.1 The *C. krusei* stock culture is now maintained lyophilized rather than frozen. The CVB-L has experienced less deterioration of the stock culture titer in the lyophilized state, but freezing is still an acceptable method of preservation.

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9. Appendices

9.1 Media formulations

9.1.1 NVSL Media Formulation No. 10204

BRAIN HEART INFUSION AGAR (BHIA)

Brain Heart Infusion Agar	52 g
QH_2O	1000 ml

Autoclave 20 min at 121°C.

9.1.2 NVSL Media formula No. 10487

TRYPTICASE SOY AGAR (TSA)
OR
SOYBEAN-CASEIN DIGEST AGAR (SCDA)

Trypticase Soy Agar	40 g
QH_2O	1000 ml

Autoclave 20 min at 121°C.

9.1.3 NVSL Media Formulation No. 10423

TRYPTICASE SOY BROTH (TSB)
or
SOYBEAN-CASEIN DIGEST MEDIUM (SCDM)

Trypticase Soy Broth	30 g
QH_2O	1000 ml

Autoclave 20 min at 121ºC.

TSB and SCDM are 2 names for the same media formulation from different media companies.

9.1.4 NVSL Media Formulation No. 10135

FLUID THIOGLYCOLLATE MEDIUM (BBL)

Fluid Thioglycollate Medium	29.5 g
QH_2O	1000 ml

Mix and heat to boiling.
Autoclave 20 min at 121°C.

9.1.5 NVSL Media Formulation No. 30035

0.15M PHOSPHATE BUFFERED SALINE with 12% SUCROSE

Potassium Phosphate Monobasic	4.7359 g
Sodium Phosphate Dibasic	16.3558 g
Sodium Chloride	8.5 g
QH_2O	QS to 1000 ml
Adjust pH to 7.3 with 10% NaOH.	
Add Sucrose.	120 g

Autoclave 20 min at 121ºC.